

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER P65141US0
		US APPLICATION NO (if known, see 37 CFR 1.5) 09/508095
INTERNATIONAL APPLICATION NO PCT/EP98/05899	INTERNATIONAL FILING DATE 16 September 1998	PRIORITY DATE CLAIMED 16 September 1997
TITLE OF INVENTION BIFIDOGENIC PEPTIDES		
APPLICANT(S) FOR DO/EO/US Hans-Dieter ZUCHT -and- Cornelia LIEPKE		

Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
 - ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

International Search Report — EPO

PCT/IB/301 Form

PCT/IB/304 Form

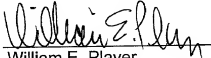
PCT/IB/308 Form

First Page of Publication

International Preliminary Examination Report — with Annexes in German

Small Entity Declaration

Sequence Listing in German

US APPLICATION NO. (if known, also 37 CFR 1.91) 097508095		INTERNATIONAL APPLICATION NO. PCT/EP98/05899		ATTORNEY'S DOCKET NUMBER P65141US0	
17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5)): Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) .. \$670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .. \$760.00 Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) \$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) \$96.00 Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) \$840.00 ENTER APPROPRIATE BASIC FEE AMOUNT = \$ 840.00				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	4 - 20 =	-0-	x \$18.00	\$	
Independent Claims	1 - 3 =	-0-	x \$78.00	\$	
Multiple Dependent Claim(s) (if applicable)			+ \$260.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$ 840.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				\$ 420.00	
SUBTOTAL =				\$ 420.00	
Processing fee of \$130 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f))				\$	
TOTAL NATIONAL FEE =				\$ 420.00	
Fee of \$40.00 for recording the enclosed assignment (37 CFR 1.21(h)). Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).				\$ 40.00	
TOTAL FEES ENCLOSED =				\$ 460.00	
				Amt. to be refunded:	\$
				Amt. charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>460.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>06-1358</u> in the amount of \$ <u>---</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. <u>06-1358</u> . A duplicate copy of this sheet is enclosed.					
SEND ALL CORRESPONDENCE TO: Jacobson, Price, Holman & Stern, PLLC 400 7th Street, N.W., Suite 600 Washington, DC 20004 202-638-6666 CUSTOMER NUMBER: 00136				By  William E. Player Reg. No. 31,409	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Hans-Dieter ZUCHT et al

Serial No.: New

Filed: March 16, 2000

For: BIFIDOGENIC PEPTIDES

PRELIMINARY AMENDMENT TO LESSEN FEES

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS

Claim 3, line 2, delete "and/or 2".

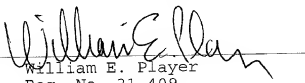
REMARKS

The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Early action on the merits is respectfully requested.

Respectfully submitted,

JACOBSON, PRICE, HOLMAN & STERN, PLLC

By 
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Date: March 16, 2000
Atty. Docket: P65141US0
WEP:jrc

Law Offices of
JACOBSON, PRICE, HOLMAN & STERN
PROFESSIONAL LIMITED LIABILITY COMPANY
THE JENIFER BUILDING
400 SEVENTH STREET, N.W.
WASHINGTON, DC 20004

Attny's Docket No. _____

SMALL ENTITY DECLARATION
[37 CFR 1.9(c-f)]

Each undersigned declares that:

(1) ☐ the application attached hereto.

(2) ☐ U.S. Application Serial No. _____, filed _____

(3) ☐ U.S. Patent No. _____ Issued _____

is entitled to the benefits of "small entity" status for paying reduced fees under 35 USC 41(a) and (b) to the Patent and Trademark Office by virtue of the following:

(4) ☒ Each undersigned declares that he/she qualifies as an independent inventor, or would qualify had he/she made the invention, as defined in 37 CFR 1.9(c).

(5) ☐ The undersigned declares that he/she is an official empowered to act on behalf of the concern identified below; that this concern qualifies as a small business concern as defined in 37 CFR 1.9(d); that exclusive rights to the invention have been conveyed to and remain with the small business concern, or if the rights are not exclusive, that all other rights belong to small entities as defined in 37 CFR 1.9.

(6) ☐ The undersigned declares that he/she is an official empowered to act on behalf of the organization identified below; that this organization qualifies as a nonprofit organization as defined in

(a) ☐ 37 CFR 1.9(e)(1)

(b) ☐ 37 CFR 1.9(e)(2)

(c) ☐ 37 CFR 1.9(e)(3)

(d) ☐ 37 CFR 1.9(e)(4) State law of _____ that exclusive rights to the invention have been conveyed to and remain with the organization, or if the rights are not exclusive, that all other rights belong to organizations as defined in 37 CFR 1.9.

(7) Each person, concern or organization to which I/we have assigned, granted, conveyed or licensed, or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

(a) ☐ no such person, concern or organization

(b) ☐ persons, concerns or organization listed below

[a separate declaration is required from each named person, concern or organization having rights to this invention averring to their status as "small entities."]

Full Name _____

Address _____

☐ Individual

☐ Small Business Concern

☐ Nonprofit Organization

I/we acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement of small entity prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I/we hereby declare all statements made herein of his/her own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application, any patent issued thereon, or any patent to which this declaration is directed.

(8) Prof. Dr. Wolf-Georg FORSSMANN Wolff-Georg 22/02/2000
Typed Name of Inventor Signature Date

Typed Name of Inventor Signature Date

Typed Name of Inventor Signature Date

Typed Name of Inventor Signature Date

(9) _____
Name of Small Business Concern or Nonprofit Organization
By _____
Typed Name Signature Date

Title of Signatory

SMB

Bifidogenic Peptides

The present invention relates to bifidogenic peptides, a process for their preparation and the use of said bifidogenic peptides.

Milk is known to promote the health of infants. This is often attributed to the influence of milk on the formation of an infant-typical intestinal flora of which more than 90% consists of *Bifidobacterium bifidum*.

It has been the object of the present invention to provide peptides which have a positive influence on the intestinal flora.

This object is achieved by peptides having the features of claim 1. The peptides according to the invention are peptides obtainable by

- adding proteases to cow's milk or human milk, followed by incubation for two hours;
- centrifugation to remove milk fat;
- acidification to a pH of 2.0 with strong acids;
- removing the precipitated proteins;
- application of at least one reverse phase HPLC step;
- application of a cation-exchange HPLC step;

- collecting fractions;
- adjusting the fractions to a salt content of < 25 mM by dialysis or reverse phase HPLC for performing activity tests;
- culturing *Bifidobacterium bifidum* and *E. coli* in the presence of the fractions and selecting fractions which meet the requirement:

$$\frac{BW}{B0} - \frac{EW}{E0} \geq 0.15 \text{ (bifidogenic)}$$

wherein BW represents the germ count obtained upon 16 hours of incubation of *Bifidobacterium bifidum* in 50% Elliker broth in the presence of the peptides in a concentration of 200 µg/ml;

B0 represents the germ count obtained in the control incubation without active substances;

EW represents the germ count obtained upon 16 hours of incubation of *E. coli* in 3 g/l tryptic soy broth in the presence of the peptides in a concentration of 200 µg/ml;

E0 represents the germ count obtained in the control incubation without active substances;

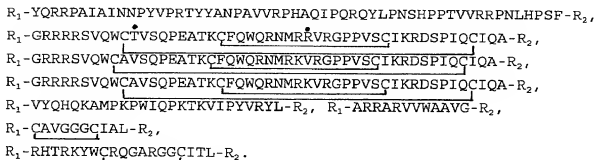
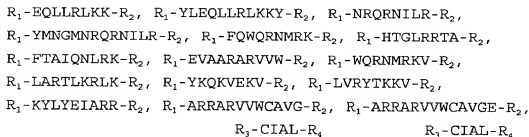
- isolation of the peptide contained in this fraction;

and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties.

The peptides according to the invention have an antimicrobial effect against bacteria which do not occur, or only so in small amounts, in the natural

infantile intestinal flora, and they promote the growth of desired bacteria, such as bifidobacteria, by promoting the growth of bifidobacteria more than that of other bacteria or by selectively inhibiting the undesired bacteria. This property of providing bifidobacteria with an advantage with respect to growth is called "bifidogenic".

Preferably, peptides are used which have the following amino acid sequence:



wherein

R_1 , R_3 independently represent NH_2 , an amino acid or a peptide containing up to 100 amino acids; and

R_2 , R_4 independently represent COOH , CONH_2 , an amino acid or a peptide containing up to 100 amino acids;

and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties.

Preferably, R_1 , R_2 , R_3 and R_4 have a length of up to 50, more preferably up to 20 and most preferably up to 10 amino acids.

The peptides according to the invention can be obtained by isolation and purification from cow's milk or human milk. Alternatively, they may also be expressed in genetically engineered organisms or prepared by chemical peptide synthesis.

Another aspect of the invention is the nucleic acids coding for the bifidogenic bacteria and antibodies directed against bifidogenic peptides.

The peptides and/or nucleic acids according to the invention can be contained in medicaments together with pharmaceutically acceptable excipients. In this case, those galenic formulations and dosage forms are selected in which the peptides reach their site of action undegraded.

Preferably, the peptides according to the invention are employed in amounts of from 0.1 to 100 mg per kg of body weight. Effective amounts of nucleic acids are, for example, from 0.01 mg to 100 mg per kg of body weight. Preferably, this amount is within a range of from 1 to 10 mg per kg of body weight for the peptides and nucleic acids.

The peptides according to the invention may also be contained in foods together with nutrients.

In addition, the peptides according to the invention and/or the antibodies directed against the peptides may also be contained in diagnostic agents together with other auxiliary agents.

The peptides and nucleic acids according to the invention are suitable for the treatment of diseases caused by misplaced microbial colonizations, such as infections, inflammations, microbially induced tumors, microbially caused

degenerative diseases, diarrheic diseases, colics, deviations in the oral, intestinal and vaginal floras, caries. The misplaced microbial colonization may be caused, for example, by bacteria, fungi, yeasts, protists, viruses, mycoplasmas, filariae and/or plasmodiums.

The peptides according to the invention are also suitable as auxiliary agents in the food preparation in terms of fermentations aids.

In particular, two or more peptides are preferably used in common, or peptides are used which have two or more of the peptide sequences according to the invention. When resistances of microorganisms occur, the different ranges of activity of the individual substances or of the substances having individual sequences of the peptides according to the invention allow to achieve an optimum inhibition of the undesired microorganisms through an appropriate combination of sequences or through a combination of individual substances.

The following Examples are intended to further illustrate the invention:

Example 1

Treatment of milk

To human milk, after having been adjusted to pH 3.5 with HCl, was added pepsin (20 mg per g of protein). The enzymatic reaction was incubated at 37 °C for two hours, and stopped by five minutes of boiling. This was followed by centrifugation (20 min, 60,000 g at 4 °C) and skimming off of the milk fat. To the resultant solution was added 0.1% TFA, and centrifugation was again performed to separate off precipitated high molecular weight proteins.

HPLC purification of a bifidogenic peptide from milk

For the purification of bifidogenic peptides from milk, several HPLC separation methods have to be combined in order to achieve preparation in as high a purity as possible through an optimum separation efficiency and to separate off inactive, undesired components. The respective samples formed after each separation step must be tested in two test systems, i.e., a growth test with bifidobacteria in combination with a growth test with *E. coli* as a target (see Examples 3 and 4). For the purification, it is necessary to combine at least one reverse phase chromatographic step (preferably two reverse phase chromatographic steps) with a cation-exchange HPLC separation. In the biotests, the respective sample must be employed in a salt-poor condition in order to obtain as optimal a screening result as possible.

The first separation step was performed by means of a Parcosil C18 column (1 x 12.5 cm, 100 Å, Biotek, Heidelberg, Germany).

Buffer A: 0.1% TFA

Buffer B: acetonitrile with 0.1% TFA

Gradient: 0 to 60% B in 45 minutes

Flow rate: 2 ml/min

Detection at 280 nm (see Figure 1)

Rechromatography of fraction 23 with the same column and a more gently rising gradient (see picture):

Buffer A: 0.1% TFA

Buffer B: acetonitrile with 0.1% TFA

Gradient: 0 to 20% B in 5 minutes

20 to 50% B in 45 minutes

Detection at 214 nm (see Figure 2)

Rechromatography of fraction 16 from the preceding separation step with the same column, but another eluent, in order to change the selectivity in the separation:

Buffer A: 0.1% TFA

Buffer B: 0.1% TFA in methanol

Gradient: 0 to 40% B in 5 minutes

40 to 70% B in 45 minutes

Detection at 214 nm (see Figure 3)

Rechromatography of the active fraction 21 by cation-exchange HPLC:

Column: Parcosil Pepkat, 4 x 50 mm, 300 Å, 5 µm, Biotek, Heidelberg

Buffer A: 10 mM phosphate buffer, pH 4.5

Buffer B: buffer A with 1 M NaCl

Flow rate: 0.75 ml/min

Gradient: 0 to 15% B in 5 minutes

15 to 50% B in 35 minutes

Detection at 214 nm (see Figure 4)

Each of the fractions obtained was separately desalted in a brief reverse phase HPLC run prior to being passed to the test for antimicrobial and bifidogenic activities.

The following peptides were identified by mass spectrometry and amino acid sequencing:

Fraction 9 contained the pure bifidogenic component:

YQRRFAIAINNPYVPRTTYANPAVVRPHAQIPQRQYLPNSHEPTVVRPRLHPSF

(casein K-63-117);

fraction 10 contained the bifidogenic component:

GRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPICCIIQA

(neutrophile lactoferrin-20-67);

and fraction 11 contains the bifidogenic component with an adduct mass of +16, which indicates that it is an oxidation product (probably, one methionine has been oxidized).

Both peptides and the oxidation product exhibit bifidogenic activity.

Example 2

Demonstration of the growth-regulating activity on E. coli

Fractions from the HPLC were employed with *E. coli* K12. The test is performed in 3 g/l tryptic soy broth (Sigma) as follows:

For each assay, cultures of *E. coli* K12 were freshly inoculated in tryptic soy broth (Sigma, Deisenhofen, Germany, order No. T8907) (Difco Manual, 10th ed., p. 1027). The incubation of these bacteria was always performed under aerobic conditions at 37 °C for 16 hours. Peptides to be tested were given to a test solution consisting of 200 µl of 3 g/l tryptic soy broth in 96-well cell culture plates, and inoculated with 20 µl of a diluted bacterial suspension. The photometric absorption of the inoculum was 0.05, meas-

ured at 500 nm. The growth of the bacteria under the influence of the peptides was also photometrically determined in an ELISA reader after 16 hours and manually determined by microscopy.

Example 3

Demonstration of the growth-regulating activity on Bifidobacterium bifidum

For each assay, cultures of *Bifidobacterium bifidum* ATCC 29521 were freshly inoculated in Elliker broth (Difco, Detroit, USA) (tryptone 20 g, yeast extract 5 g, gelatin 2.5 g, dextrose 5 g, lactose 5 g, saccharose 5 g, sodium chloride 4 g, sodium acetate 1.5 g, ascorbic acid 0.5 g). The incubation of these bacteria was always performed under anaerobic conditions at 37 °C for 16 to 18 hours. Peptides to be tested were given to a test solution consisting of 200 µl of 50% Elliker broth in 96-well cell culture plates, and inoculated with 20 µl of a diluted bacterial suspension. The photometric absorption of the inoculum was 0.05, measured at 550 nm. The growth of the bacteria under the influence of the peptides was also photometrically determined in an ELISA reader after 16 hours and manually determined by microscopy. N-Acetylglucosamine served as a positive control. Only bifidus cultures which respond to N-acetylglucosamine can be used for this test. After some passages, bifidobacteria lose this property; in this case, they can no longer be used for this growth test.

Example 4

Those fractions in which the value

$$\frac{BW}{B0} - \frac{EW}{E0} \geq 0.15 \text{ (bifidogenic)}$$

were identified as being bifidogenic.

ART 34 ANDY

CLAIMS:

(amended November 2, 1999)

1. Use of peptides obtainable by

- adding proteases to cow's milk or human milk, followed by incubation for two hours;
- centrifugation to remove milk fat;
- acidification to a pH of 2.0 with strong acids;
- removing the precipitated proteins;
- application of at least one reverse phase HPLC step;
- application of a cation-exchange HPLC step;
- collecting fractions;
- adjusting the fractions to a salt content of < 25 mM by dialysis or reverse phase HPLC for performing activity tests;
- culturing *Bifidobacterium bifidum* and *E. coli* in the presence of the fractions and selecting fractions which meet the requirement:

$$\frac{BW}{B0} - \frac{EW}{E0} \geq 0.15 \text{ (bifidogenic)}$$

wherein BW represents the germ count obtained upon 16 hours of incubation of *Bifidobacterium bifidum* in 50% Elliker broth in the presence of the peptides in a concentration of 200 µg/ml;

B0 represents the germ count obtained in the control incubation without active substances;

EW represents the germ count obtained upon 16 hours of incubation of *E. coli* in 3 g/l tryptic soy broth in the presence of the peptides in a concentration of 200 µg/ml;

E0 represents the germ count obtained in the control incubation without active substances;

- isolation of the peptide contained in this fraction;

and of the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties, and of peptides obtainable by the combination of the peptides, fragments or derivatives by chemical bonding, for the preparation of a medicament for the treatment of diseases caused by misplaced microbial colonizations, for example, by bacteria, fungi, yeasts, protists, viruses, mycoplasmas, filariae, plasmodiums, such as infections, inflammations, microbially induced tumors, microbially caused degenerative diseases, diarrhetic diseases, colics, deviations in the oral, intestinal and vaginal floras, caries.

2. The use according to claim 1 wherein peptides are used having the amino acid sequence:

R_1 -EQLLRLKK- R_2 , R_1 -YLEQLRLKKY- R_2 , R_1 -NRQRNLR- R_2 ,
 R_1 -YMNGMNRQRNLR- R_2 , R_1 -FQQRNMRK- R_2 , R_1 -HTGLRRTA- R_2 ,
 R_1 -FTAIQNLRK- R_2 , R_1 -EVAARARVVW- R_2 , R_1 -WQRNMRKV- R_2 ,
 R_1 -LARTLKRLK- R_2 , R_1 -YKQKVEKV- R_2 , R_1 -LVRYTKKV- R_2 ,
 R_1 -KYLVEIARR- R_2 , R_1 -ARRARVVWCAVG- R_2 , R_1 -ARRARVVWCAVGE- R_2 ,
 R_3 -CIAL- R_4 R_3 -CIAL- R_4

R_1 -YQRRPAIAINNPFYVPRYYANPAVVRPHAQIPQRQYLPNSHPPTVVRRPNLHPSF- R_2 ,
 R_1 -GRRRRSVQWCTVSQPEATKCFQWQRNMRVRGPPVSCIKRDSPIQCIQA- R_2 ,
 R_1 -GRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQA- R_2 ,
 R_1 -GRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQA- R_2 ,
 R_1 -VYQHQQAMPKPWIQPKTKVIPYVRYL- R_2 , R_1 -ARRARVVWAAVG- R_2 ,
 R_1 -CAVGGGCIAL- R_2 ,
 R_1 -RHTRKYWCRCQARGGCITL- R_2 .

wherein

R_1 , R_3 independently represent NH_2 , an amino acid or a peptide containing up to 100 amino acids; and

R_2 , R_4 independently represent $COOH$, $CONH_2$, an amino acid or a peptide containing up to 100 amino acids;

and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties.

- Use of nucleic acids coding for the peptides mentioned in claim 1 and/or 2 for the preparation of a medicament for the treatment of diseases caused by misplaced microbial colonizations, for example, by bacteria, fungi, yeasts, protists, viruses, mycoplasmas, filariae, plasmodiums, such as infections, inflammations, microbially induced tu-

mors, microbially caused degenerative diseases, diarrheic diseases, colics, deviations in the oral, intestinal and vaginal floras, caries.

4. Peptides having the SEQ ID Nos. 10, 22 and 23 for the use according to claim 1.

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Abstract

Peptides obtainable by

- adding proteases to cow's milk or human milk, followed by incubation for two hours;
- centrifugation to remove milk fat;
- acidification to a pH of 2.0 with strong acids;
- removing the precipitated proteins;
- application of at least one reverse phase HPLC step;
- application of a cation-exchange HPLC step;
- collecting fractions;
- adjusting the fractions to a salt content of < 25 mM by dialysis or reverse phase HPLC for performing activity tests;
- culturing *Bifidobacterium bifidum* and *E. coli* in the presence of the fractions and selecting fractions which meet the requirement:

$$\frac{BW}{BO} - \frac{EW}{EO} \geq 0.15 \text{ (bifidogenic)}$$

wherein BW represents the germ count obtained upon 16 hours of incubation of *Bifidobacterium bifidum* in 50% Elliker broth in the presence of the peptides in a concentration of 200 µg/ml;

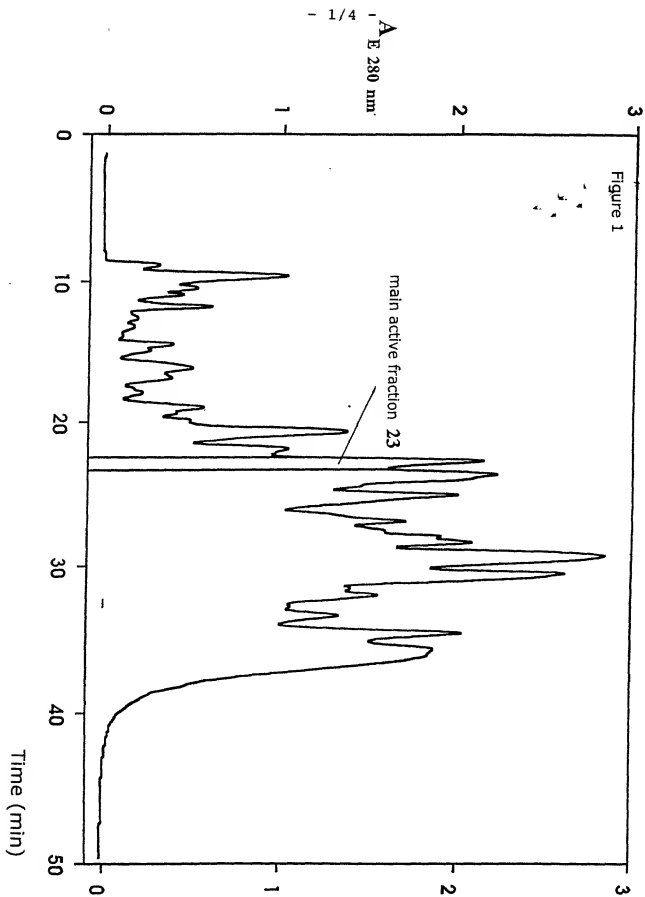
B0 represents the germ count obtained in the control incubation without active substances;

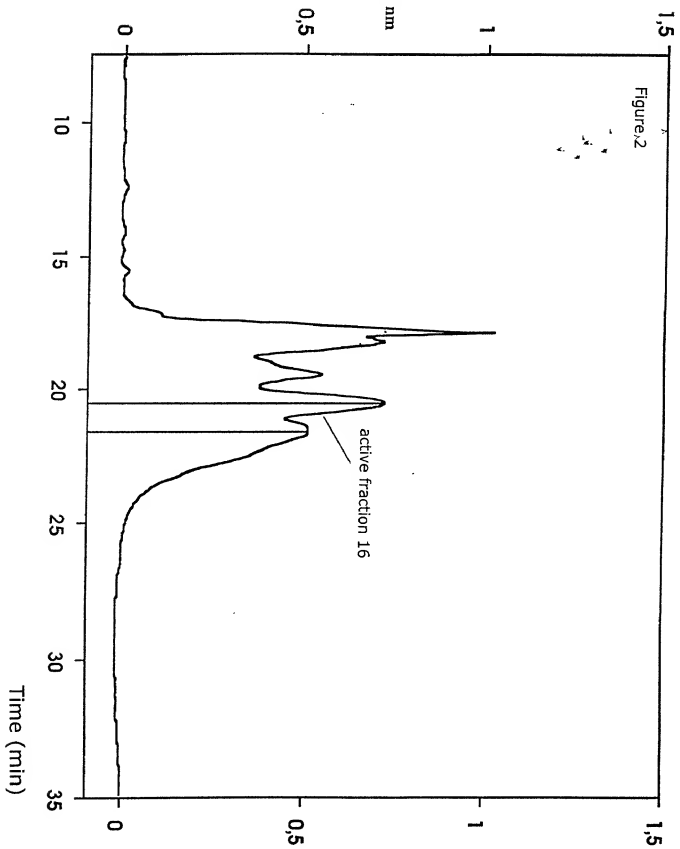
EW represents the germ count obtained upon 16 hours of incubation of *E. coli* in 3 g/l tryptic soy broth in the presence of the peptides in a concentration of 200 µg/ml;

E0 represents the germ count obtained in the control incubation without active substances;

- isolation of the peptide contained in this fraction;

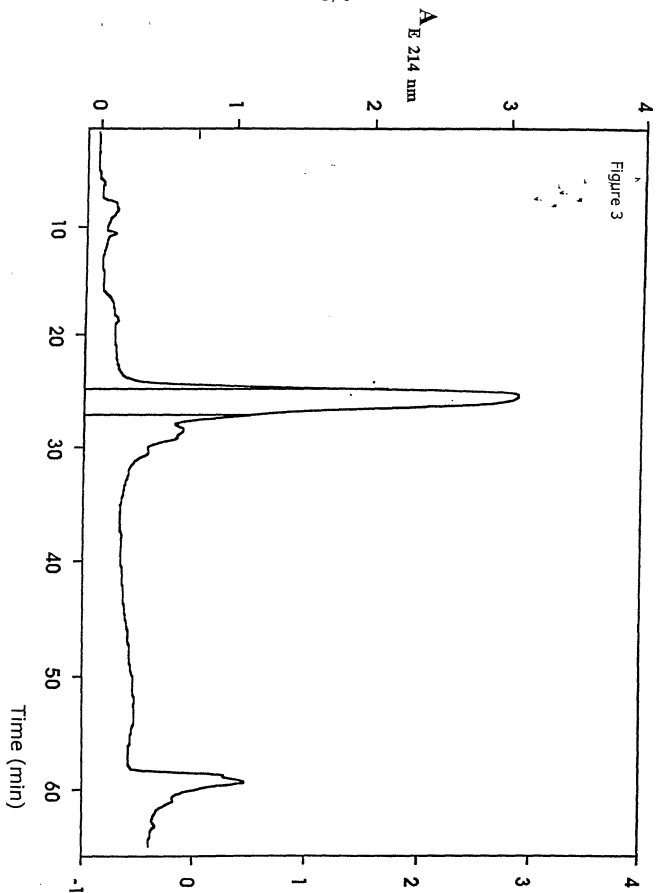
and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties, and peptides obtainable by the combination of the peptides, fragments or derivatives by chemical bonding.



$\frac{2}{4}$
A
E 280 nm

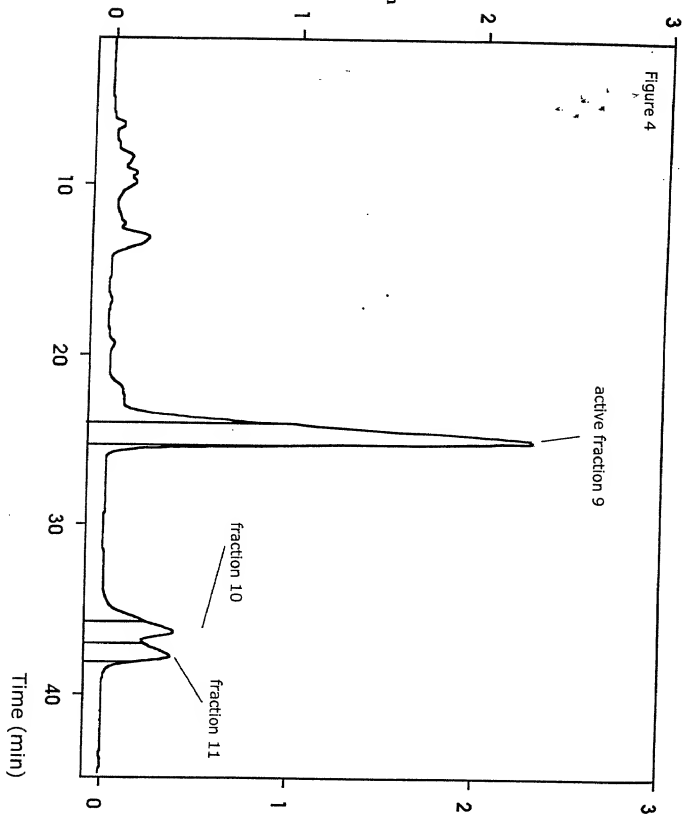
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$A_{E 214 \text{ nm}}$
- 4/4



- 10 -

SEQUENZPROTOKOLL

(1) ALLGEMEINE ANGABEN:

- (i) ANMELDER:
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 - (B) STRASSE: Feodor-Lynen-Strasse 31
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 - (F) POSTLEITZAHL: 30625
- (ii) BEZEICHNUNG DER ERFINDUNG: Bifidogene Peptide
- (iii) ANZAHL DER SEQUENZEN: 24
- (iv) COMPUTER-LESBARE FASSUNG:
 - (A) DATENTRÄGER: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) BETRIEBSSYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPA)

(2) ANGABEN ZU SEQ ID NO: 1:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 8 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt
- (ii) ART DES MOLEKÜLS: Peptid
- (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 1:

Glu Gln Leu Leu Arg Leu Lys Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 2:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 11 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt
- (ii) ART DES MOLEKÜLS: Peptid
- (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 2:

Tyr Leu Glu Gln Leu Leu Arg Leu Lys Lys Tyr
1 5 10

(2) ANGABEN ZU SEQ ID NO: 3:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 8 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt
- (ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 3:

Asn Arg Gln Arg Asn Ile Leu Arg
1 5

(2) ANGABEN ZU SEQ ID NO: 4:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 13 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 4:

Tyr Met Asn Gly Met Asn Arg Gln Arg Asn Ile Leu Arg
1 5 10

(2) ANGABEN ZU SEQ ID NO: 5:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 9 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 5:

Phe Gln Trp Gln Arg Asn Met Arg Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 6:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 8 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 6:

His Thr Gly Leu Arg Arg Thr Ala
1 5

(2) ANGABEN ZU SEQ ID NO: 7:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 9 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 7:

Phe Thr Ala Ile Gln Asn Leu Arg Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 8:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 10 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 8:

Glu Val Ala Ala Arg Ala Arg Val Val Trp
1 5 10

(2) ANGABEN ZU SEQ ID NO: 9:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 8 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 9:

Trp Gln Arg Asn Met Arg Lys Val
1 5

(2) ANGABEN ZU SEQ ID NO: 10:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 9 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 10:

Leu Ala Arg Thr Leu Lys Arg Leu Lys
1 5

(2) ANGABEN ZU SEQ ID NO: 11:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 8 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 11:

Tyr Lys Gln Lys Val Glu Lys Val
1 5

(2) ANGABEN ZU SEQ ID NO: 12:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 8 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 12:

Leu Val Arg Tyr Thr Lys Lys Val
1 5

(2) ANGABEN ZU SEQ ID NO: 13:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 9 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 13:

Lys Tyr Leu Tyr Glu Ile Ala Arg Arg
1 5

(2) ANGABEN ZU SEQ ID NO: 14:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 12 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 14:

Ala Arg Arg Ala Arg Val Val Trp Cys Ala Val Gly
1 5 10

(2) ANGABEN ZU SEQ ID NO: 15:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 4 Aminosäuren
- (B) ART: Aminosäure
- (C) STRANGFORM: nicht bekannt
- (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 15:

Cys Ile Ala Leu
1

(2) ANGABEN ZU SEQ ID NO: 16:

- (i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 13 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 16:

Ala Arg Arg Ala Arg Val Val Trp Cys Ala Val Gly Glu
1 5 10

(2) ANGABEN ZU SEQ ID NO: 17:

- (i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 55 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 17:

Tyr Gln Arg Arg Pro Ala Ile Ala Ile Asn Asn Pro Tyr Val Pro Arg
1 5 10 15

Thr Tyr Tyr Ala Asn Pro Ala Val Val Arg Pro His Ala Gln Ile Pro
20 25 30

Gln Arg Gln Tyr Leu Pro Asn Ser His Pro Pro Thr Val Val Arg Arg
35 40 45

Pro Asn Leu His Pro Ser Phe
50 55

(2) ANGABEN ZU SEQ ID NO: 18:

- (i) SEQUENZKENNZEICHEN:
(A) LÄNGE: 49 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 18:

Gly Arg Arg Arg Arg Ser Val Gln Trp Cys Thr Val Ser Gln Pro Glu
1 5 10 15

Ala Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Arg Val Arg Gly
20 25 30

Pro Pro Val Ser Cys Ile Lys Arg Asp Ser Pro Ile Gln Cys Ile Gln
35 40 45

Ala

(2) ANGABEN ZU SEQ ID NO: 19:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 48 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 19:

Gly Arg Arg Arg Ser Val Gln Trp Cys Ala Val Ser Gln Pro Glu Ala
1 5 10 15
Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Lys Val Arg Gly Pro
20 25 30
Pro Val Ser Cys Ile Lys Arg Asp Ser Pro Ile Gln Cys Ile Gln Ala
35 40 45

(2) ANGABEN ZU SEQ ID NO: 20:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 49 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 20:

Gly Arg Arg Arg Arg Ser Val Gln Trp Cys Ala Val Ser Gln Pro Glu
1 5 10 15
Ala Thr Lys Cys Phe Gln Trp Gln Arg Asn Met Arg Lys Val Arg Gly
20 25 30
Pro Pro Val Ser Cys Ile Lys Arg Asp Ser Pro Ile Gln Cys Ile Gln
35 40 45

Ala

(2) ANGABEN ZU SEQ ID NO: 21:

- (i) SEQUENZKENNZEICHEN:
 - (A) LÄNGE: 26 Aminosäuren
 - (B) ART: Aminosäure
 - (C) STRANGFORM: nicht bekannt
 - (D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 21:

Val Tyr Gln His Gln Lys Ala Met Pro Lys Pro Trp Ile Gln Pro Lys
1 5 10 15

Thr Lys Val Ile Pro Tyr Val Arg Tyr Leu
20 25

(2) ANGABEN ZU SEQ ID NO: 22:

(i) SEOUENZKENNZEICHEN:

- (A) LÄNGE: 12 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 22:

Ala Arg Arg Ala Arg Val Val Trp Ala Ala Val Gly
1 5 10

(2) ANGABEN ZU SEQ ID NO: 23:

(i) SEOUENZKENNZEICHEN:

- (A) LÄNGE: 10 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 23:

Cys Ala Val Gly Gly Gly Cys Ile Ala Leu
1 5 10

(2) ANGABEN ZU SEQ ID NO: 24:

(i) SEQUENZKENNZEICHEN:

- (A) LÄNGE: 19 Aminosäuren
(B) ART: Aminosäure
(C) STRANGFORM: nicht bekannt
(D) TOPOLOGIE: nicht bekannt

(ii) ART DES MOLEKÜLS: Peptid

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 24:

Arg His Thr Arg Lys Tyr Trp Cys Arg Gln Gly Ala Arg Gly Gly Cys
1 5 10 15

Ile Thr Leu

DECLARATION AND POWER OF ATTORNEY U.S.A.

FOR ATTORNEY'S USE ONLY
ATTORNEY'S DOCKET NO. _____

ALL PATENTS, INCLUDING DESIGN
FOR APPLICATION BASED ON PCT, PARIS CONVENTION,
NON PRIORITY, OR PROVISIONAL APPLICATIONS

As a below named inventor, I declare that my residence, post office address and citizenship are stated below next to my name, the information given herein is true, that I believe that I am the original, first and sole inventor (if only one name is listed at 201 below), or a first and joint inventor (if plural inventors are named below at 201-203, or on additional sheets attached hereto) of the subject matter which is claimed and for which patent is sought on the invention entitled:

Bifidogenic Peptides

which is described and claimed in, ☒ PCT International Application No. PCT/EP 98/05899 filed 16/09/1998
☐ the attached specification ☐ the specification in application (if applicable) and _____ filed _____

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above
I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56
I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed
<u>197 40 604.1</u>	<u>Germany</u>	<u>16/09/1997</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
(Number)	(Country)	(Day/Month/Year Filing)	
<u>198 05 385.1</u>	<u>Germany</u>	<u>11/02/1998</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
(Number)	(Country)	(Day/Month/Year Filing)	
(Number)	(Country)	(Day/Month/Year Filing)	<input type="checkbox"/> Yes <input type="checkbox"/> No

I hereby claim the benefit under Title 35, United States Code, §110(e) of any United States provisional application(s) listed below:

Application No. _____ Filing Date _____ Application No. _____ Filing Date _____

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status: patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys (Registration No.) to prosecute this application, receive and act on instructions from my agent, and transact all business in the Patent and Trademark Office connected therewith. HARVEY B. JACOBSON JR. (20,854)-D. DOUGLAS PRICE (24,514); JOHN CLARKE HOLMAN (22,769); MARVIN R. STERN (20,640); MICHAEL R. SLOBASKY (26,421); JONATHAN L. SCHERER (29,851); IRWIN M. AISENBERG (19,007); WILLIAM E. PLAYER (31,409)

<p>SEND CORRESPONDENCE TO:</p> <p>JACOBSON, PRICE, HOLMAN & STERN PROFESSIONAL LIMITED LIABILITY COMPANY 400 SEVENTH STREET N.W. WASHINGTON, DC 20004</p>	<p>DIRECT TELEPHONE CALLS TO:</p> <p>(please use Attorney's Docket No.) (202) 638-6666</p> <p>JACOBSON, PRICE, HOLMAN & STERN PROFESSIONAL LIMITED LIABILITY COMPANY</p>
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Inventor(s) name must include at least one unabbreviated first or middle name.

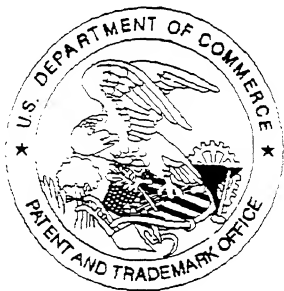
201	<p>FULL NAME OF INVENTOR * <u>WOLF-Georg</u></p> <p>RESIDENCE & CITIZENSHIP <u>City Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>FAMILY NAME <u>WOLF-Georg</u></p> <p>CITY <u>Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>GIVEN NAME <u>WOLF-Georg</u></p> <p>STATE OR FOREIGN COUNTRY <u>Germany</u></p> <p>CITY <u>Hannover</u></p>	<p>MIDDLE NAME <u>DEX</u></p> <p>COUNTRY OF CITIZENSHIP <u>Germany</u></p> <p>STATE OR COUNTRY <u>Germany</u></p> <p>ZIP CODE <u>30625</u></p>
202	<p>FULL NAME OF INVENTOR * <u>ZUCHI</u></p> <p>RESIDENCE & CITIZENSHIP <u>City Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>FAMILY NAME <u>ZUCHI</u></p> <p>CITY <u>Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>GIVEN NAME <u>Hans-Dieter</u></p> <p>STATE OR FOREIGN COUNTRY <u>Germany</u></p> <p>CITY <u>Hannover</u></p>	<p>MIDDLE NAME <u>DEX</u></p> <p>COUNTRY OF CITIZENSHIP <u>Germany</u></p> <p>STATE OR COUNTRY <u>Germany</u></p> <p>ZIP CODE <u>30625</u></p>
203	<p>FULL NAME OF INVENTOR * <u>LEPKE</u></p> <p>RESIDENCE & CITIZENSHIP <u>City Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>FAMILY NAME <u>LEPKE</u></p> <p>CITY <u>Hannover</u></p> <p>POST OFFICE ADDRESS <u>Feodor-Lynen-Str. 31</u></p>	<p>GIVEN NAME <u>Cornelia</u></p> <p>STATE OR FOREIGN COUNTRY <u>Germany</u></p> <p>CITY <u>Hannover</u></p>	<p>MIDDLE NAME <u>DEX</u></p> <p>COUNTRY OF CITIZENSHIP <u>Germany</u></p> <p>STATE OR COUNTRY <u>Germany</u></p> <p>ZIP CODE <u>30625</u></p>

I further declare that all statements made herein of my own knowledge are true and that all statement made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon

<p>SIGNATURE OF INVENTOR 201 <u>WOLF-Georg</u></p> <p>DATE <u>22/02/2000</u></p>	<p>SIGNATURE OF INVENTOR 202 <u>Hans-Dieter ZUCHI</u></p> <p>DATE <u>22/02/2000</u></p>	<p>SIGNATURE OF INVENTOR 203 <u>Cornelia LEPKE</u></p> <p>DATE <u>22/02/2000</u></p>
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The ABSTRACT is Numbered pgs. 14 & 15.
Pages 5-13 - NOT in CASE.